The Effects of Electron and Chemical Ionization Modes on the MS Profiling of Whole Bacteria

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Free fatty acid profiling of whole bacteria [*Francisella tularensis*, *Brucella melitensis*, *Yersinia pestis*, *Bacillus anthracis* (vegetative and sporulated), and *Bacillus cereus*] was carried out with direct probe mass spectrometry under 70-eV electron ionization (EI) and isobutane chemical ionization in both the positive (CI^+) and negative modes (CI^-). Electron ionization produced spectra that contained molecular ions and fragment ions from various free fatty acids. Spectra acquired with isobutane chemical ionization in the positive mode yielded molecular ions of free fatty acids as well as ions from other bacterial compounds not observed under EI conditions. Spectra obtained with negative chemical ionization did not contain as much taxonomic information as EI or CI^+; however, some taxonomically significant compounds such as dipicolinic acid and poly(3-hydroxybutyrate) did produce negative ions. All ionization modes yielded spectra that could separate the bacteria by Gram-type when observed with principle components analysis (PCA). Chemical ionization in the positive ion mode produced the greatest amount of differentiation between the four genera of bacteria when the spectra where examined by PCA. (J Am Soc Mass Spectrom 1999, 10, 747–758)

Mass spectrometry has been applied to microbiology for decades, with the aim of providing a rapid technique for the analysis of microorganisms [1–5]. In order to take advantage of the rapid analysis that mass spectrometry provides, however, bacterial samples must be prepared for mass analysis quickly and simply. The simplest methods do not involve extraction or isolation of significant bacterial components, but interrogate all of the bacteria's chemical constituents simultaneously by analyzing the whole bacteria directly. Thermal methods, such as pyrolysis and thermal desorption, have traditionally provided a quick and straightforward means of preparing whole microorganisms for mass spectral analysis. Such methods are well suited to applications that require speed and simplicity, such as in field-portable instruments [6–8].

The majority of the pyrolysis or thermal desorption studies on whole bacteria have been conducted using EI at either 15 or 70 eV. Seventy-eV conditions have been used so that the spectra from whole bacteria could be more easily compared to standard spectra while 15-eV conditions have been utilized to decrease the complexity of the spectra by minimizing EI fragmentation. A review of the literature reveals that only a few papers have described the direct analysis of whole bacteria using positive and/or negative chemical ionization mass spectrometry [9–13]. In one such study by Tas et al., whole bacteria were examined with pyrolysis using EI, CI^+, and direct chemical ionization (DCI) MS methods of analysis [12]. This study found that DCI produced the greatest amount of taxonomic information. While this work only examined two genera of bacteria and no emphasis was placed on identifying the diagnostic peaks in the spectra, it was important in demonstrating the potential benefits of chemical ionization MS to bacterial taxonomy. Despite the indications that chemical ionization mass spectra have more bacterial discriminating power, little has been done with whole bacteria analysis using chemical ionization since these initial studies.

In order to thoroughly assess the effects of chemical ionization on direct bacterial profiling, a set of bacteria was examined under 70-eV EI, CI^+ and CI^- modes. Particular attention was placed on the bacterial lipids, since lipid profiling is a common chemotaxonomic procedure used to identify bacteria [4, 14–17]. Previous work using pyrolysis MS with 70-eV EI conditions has

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Table 1. Bacteria used in ionization mode comparison study and in PCA plots

Bacteria used for PCA	Media	Gram type	PCA symbol
Bacillus anthracis—sterne	CAD	+	A
Bacillus anthracis—Zimbabwe (Spores)	CAD	+	S
Bacillus cereus ATCC 11778	CAD	+	C
Brucella melitensis—abortus/WILD	BB	−	B
Yersinia pestis—La Paz	TSB	−	Y
Francisella tularensis—Palaeartica	MHB	−	F

demonstrated that direct MS lipid profiling of whole bacteria is comparable to the traditional chromatographic procedure yet is simpler and requires less time for the analysis [18].

Experimental

All spectra were collected on a JEOL JMS-700T magnetic sector instrument using a direct solids probe. The ion source was kept at 200 °C and the solids probe was heated from 30 to 300 °C at 128 °C/min for 4 min to thermally desorb the sample. A 4-μL aqueous suspension of bacteria at 10 mg/mL was used for all analyses except where noted. Bacterial samples were placed into glass capillary tubes and air dried before introduction into the mass spectrometer. Samples were analyzed directly without any prior treatment. EI data was collected at 70 eV and CI data at 200 eV over a mass range of 165–425 Da. This mass range was selected to include the molecular ions of the known free fatty acids present in these bacteria. Isobutane chemical ionization in both positive and negative modes was conducted at a source pressure of 1×10^{-3} Pa. The bacteria used for the ionization mode comparison study are summarized in Table 1. Table 2 contains bacteria that were further examined to assess the effects of growth conditions and strain variation on the diagnostic ions in their mass spectra. *B. anthracis*, *B. cereus*, *F. tularensis*, *B. melitensis* and *Y. pestis* were obtained from the Armed Forces Institute of Pathology as lyophilized and gamma killed samples. Bacterial samples were washed of growth media by centrifuging cultures at 7000 RPM for 10 min, pouring off the supernatant and re-suspending in sterile saline solution. This procedure was repeated twice. Solvent extractions of lipids from *B. melitensis* were performed according to Ref 19.

Table 2. Bacteria examined to assess the effects of growth conditions and bacterial strain on identified diagnostic ions

Bacteria used for biomarker study	Media
Francisella tularensis	
LVS Vaccine	MH
Type A Utah 2173	MH
Type A Utah 2173	CAD
Palaeartica	MHB
Brucella melitensis	
Abortus	BB
Suis	BB
Brucella neotomae	BB
Yersinia pestis	
195/p India	TSB
Nairobi, Kenya	BA
La Paz	TSB
A1122 California	BA

BA = Brucella Agar, BB = Brucella Broth, CAD = Casein Acid Digest, MH = Mueller Hinton, MHB = Mueller Hinton Broth, TSB = Trypticase Soy Broth.

Principal Components Analysis

Principal components analysis (PCA) was employed to examine the similarity of the spectra gathered under each ionization mode. Score plots of the principle components allowed the effect of each ionization mode on the bacterial profiles to be visually compared. Principal components analysis was conducted with the RESOLVE program developed at the Colorado School of Mines using all peaks in the selected mass range [20]. All spectra were mean centered and normalized to total intensity prior to PCA. For each ionization mode, spectra used for PCA were collected over a 24-h period and in random order with a minimum of four replicates per sample. All replicates were included in the PCA and no outliers were removed. In all but one case, a combination of the first two principal components produced the greatest amount of separation between the clusters of different species of bacteria.

Results

EI Positive Spectra

The direct probe EI spectra of the bacteria (Figure 1A–D) were composed of peaks from various free fatty acid molecular ions and fragment ions. These ions are summarized in Table 3. As observed in other lipid analyses of these bacteria, palmitic acid, C16:0 (*m/z* 256), was the most prevalent free fatty acid (FFA) [21–23]. Palmitic acid appeared in the EI spectra of all the bacteria and was the base peak in *B. cereus* vegetative, *B. anthracis* vegetative, *F. tularensis*, and *Y. pestis*.

The spectra from the three *Bacillus* samples (*B. anthracis* sporulated and *B. cereus* not shown) were qualitatively very similar to one another. All contained prominent peaks from C15:0 (*m/z* 242), C16:0 (*m/z* 256), C17:0 (*m/z* 270), and C18:0 (*m/z* 284) FFAs, which is in agreement with their lipid profiles as obtained by other methods [21]. Small differences in the spectra of the three *Bacillus* samples were a result of differences in the relative intensities of the major fatty acid peaks. The spectra of the sporulated *B. anthracis* were different from that of *B. anthracis* vegetative and *B. cereus* in that C15:0 FFA was the base peak in the sporulated samples.

In accordance with their established free fatty acid composition, *B. melitensis* (Figure 1C), *F. tularensis* (Figure 1D), and *Y. pestis* (Figure 1B) produced a peak at *m/z*

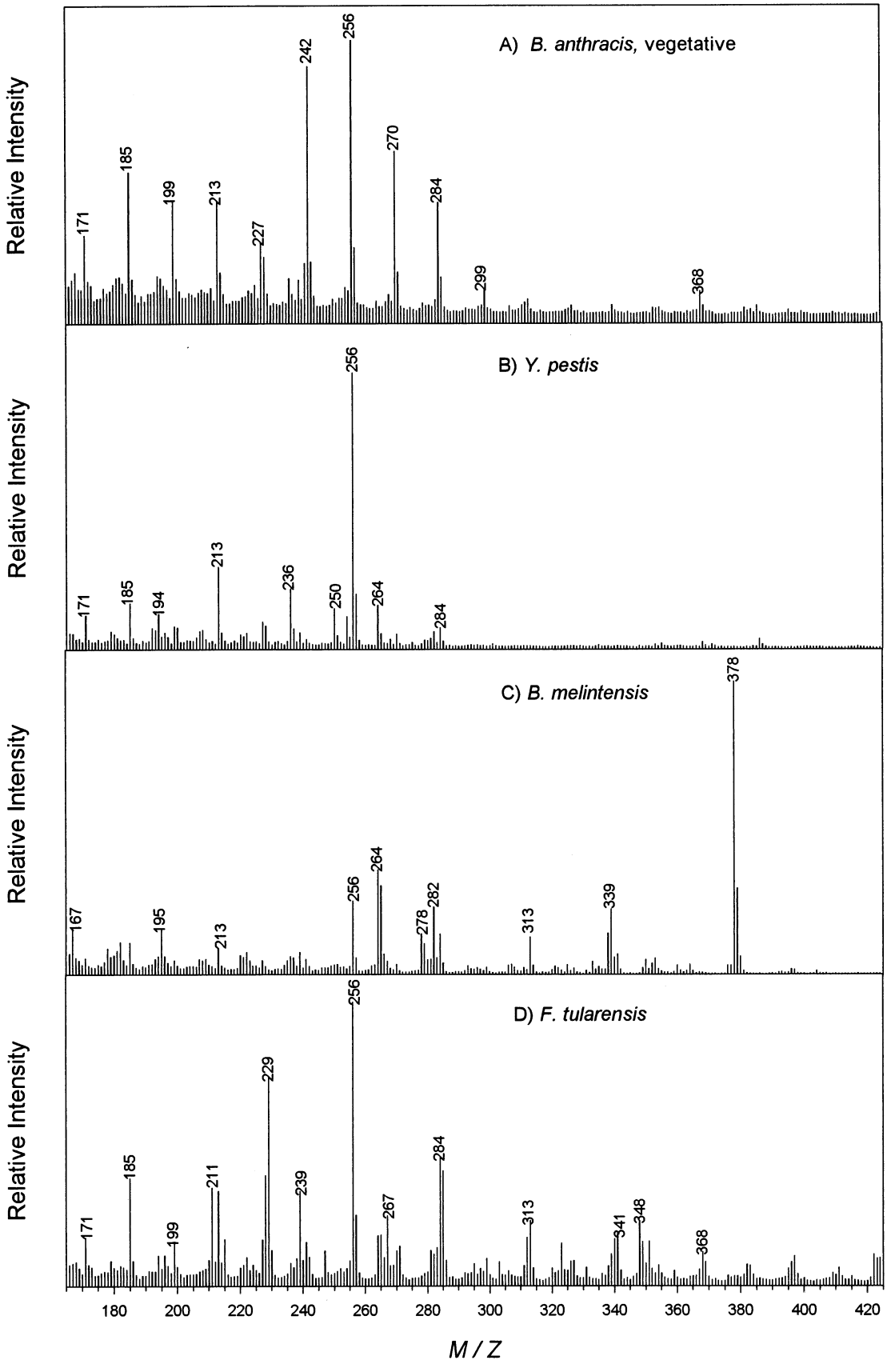

Figure 1. 70-eV EI spectra of selected whole bacteria.

Table 3. Common EI fatty acid molecular ions and their fragments

Acid	*m/z*	Acid	*m/z*	Acid	*m/z*
C10:0	172	C16:1	254	C21:0	326
C10:1	170	M-18	236		
				C22:0	340
C12:0	200	C17:0	270	C22:1	338
C12:1	198	C17:1	268	M-18	320
		M-18	250		
C13:0	214			C23:0	354
		cyC17:0	268		
C14:0	228			C24:0	368
		C18:0	284	C24:1	366
C15:0	242	C18:1	282	M-18	348
M-29	213	M-18	264		
C15:1	240				
M-18	222	C19:0	298		
		C19:1	296		
C16:0	256	M-18	278		
M-29	227				
−14	213	cyC19:0	296		
−14	199	M-18	278		
−14	185				
−14	171	C20:0	312		

CX:Y signifies carbon length of fatty acid where *X* = number of carbons in fatty acid and *Y* = number of unsaturations. The prefix "cy" indicates a cyclopropyl fatty acid.

264 corresponding to the M-18 ion of C18:1 FFA (*m/z* 282) and a peak at *m/z* 284 from C18:0 FFA [23–25]. Product-ion experiments confirmed that the ion at *m/z* 339 was a C18:1 glyceride fragment ion. The ion at *m/z* 378 was only observed in the spectra of *B. melitensis* and not in the spectra of the other bacteria (Figure 1C). The exact identity of the ion at *m/z* 378 is unknown; however, it was observed in the lipid fraction of a whole cell solvent extraction of *B. melitensis*. Both *m/z* 339 and *m/z* 378 were present in all the *Brucella* strains examined (Table 2).

The spectra of *F. tularensis* included several fatty acid peaks not observed in the spectra of the other bacteria. These peaks corresponded to the C22:0 (*m/z* 340) and C24:0 (*m/z* 368) FFAs which have been found to be unique to *F. tularensis* [24, 26]. In addition to these fatty acids, there was a large C15:0 glyceride peak at *m/z* 229. A significant C15:0 glyceride peak in the pyrolysis mass spectrum of *F. tularensis* has previously been reported by Synder et al. [27].

Y. pestis also contained fatty acid peaks not present in the other bacteria. Unlike the other bacteria, the spectra of *Y. pestis* contained peaks at *m/z* 236 and *m/z* 250 associated with the M-18 ions of C16:1 and cyC17:0 FFAs, respectively. These two fatty acids have been shown to be present in significant amounts in *Y. pestis* [23].

The PCA score plot of the mass spectra obtained with EI produced four distinct clusters with the spectra of the *Bacillus* samples (*B. anthracis* spores, *B. anthracis* vegetative, and *B. cereus* vegetative, S, A, and C respectively) forming one group (Figure 2A). Although the

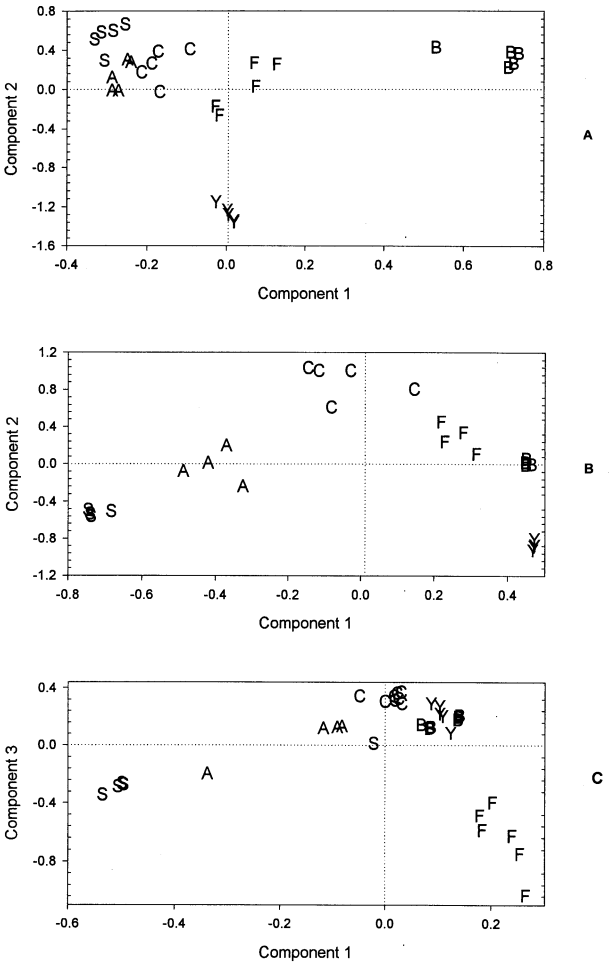

Figure 2. PCA score plots of whole bacterial mass spectra under (**A**) 70-eV EI conditions, (**B**) isobutane chemical-ionization, positive mode, (**C**) isobutane chemical-ionization, negative mode. In all PCA plots **F** = *F. tularensis*, **Y** = *Y. pestis*, **A** = *B. anthracis* (vegetative), **S** = *B. anthracis* (sporulated), **C** = *B. cereus*, **B** = *B. melitensis*.

Bacillus species were not separated from one another, Gram-type differentiation was demonstrated along the Component 1 score axis. The peaks responsible for grouping the Gram-positive species together are located in the bottom half of the Component 1 loading plot (Figure 3A). The bottom half of the loading plot corresponds to samples with negative scores (S, C, & A) along the Component 1 score axis. Similarly, samples with positive scores (F, Y, & B) along the Component 1 score axis are correlated with peaks in the upper half of the loading plot (Figure 2A). As observed in previous work, the primary ion responsible for separating the *Bacillus* spectra from the rest of the bacteria was *m/z* 242 corresponding to C15:0 FFA [17, 18]. All of the peaks in the loading plot were associated with free fatty acids except for the ions at *m/z* 339 and *m/z* 378 observed in *B. melitensis*. Component 1 contained 34% of the total variance and Component 2 contained 28%.

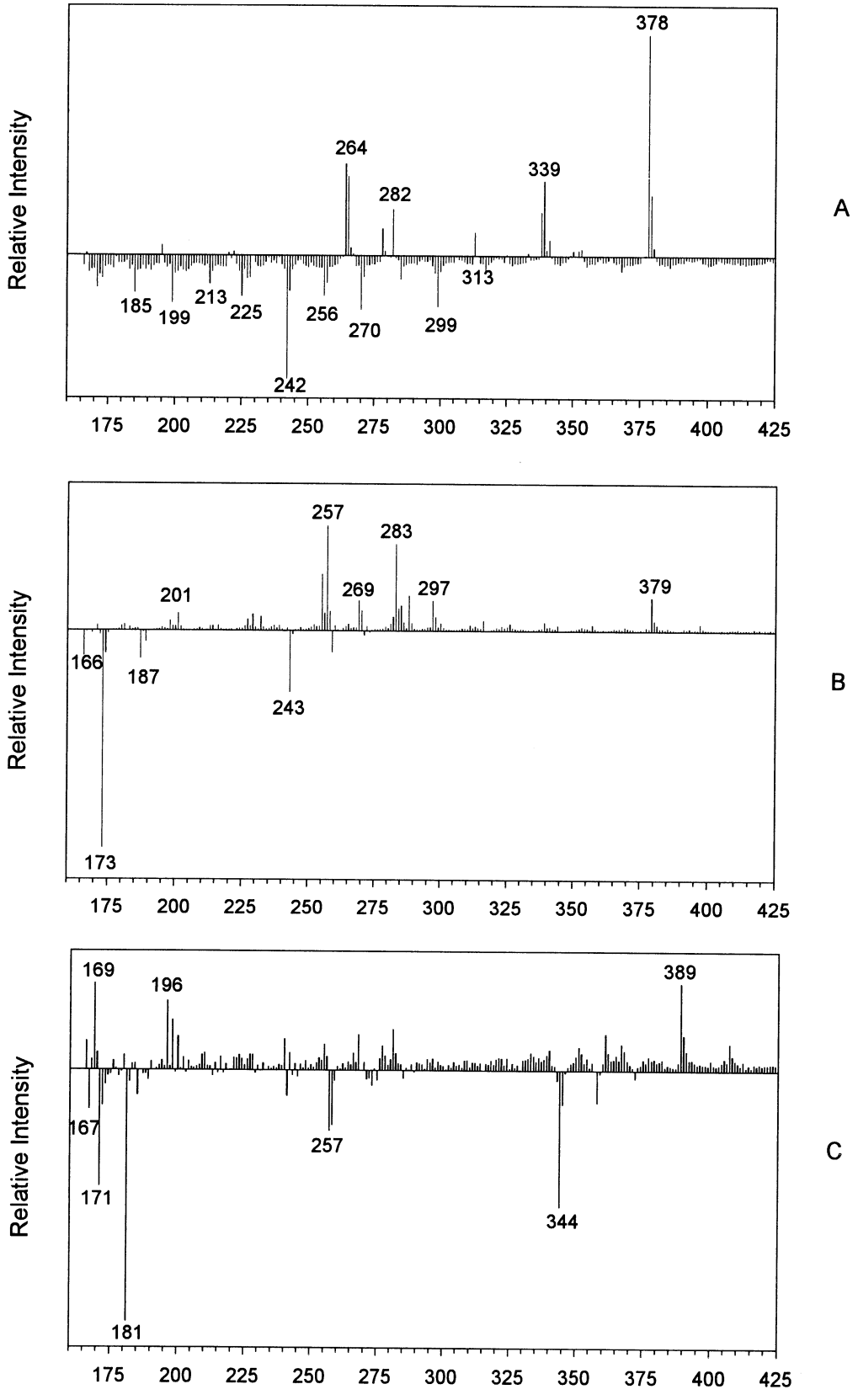

Figure 3. Component 1 loadings for the PCA score plots shown in Figure 2. Component 1 from (**A**) EI, (**B**) CI^+, (**C**) CI^- data. In all loadings spectra from *Y. pestis*, *F. tularensis*, and *B. melitensis* fall above the component axis and spectra from the *Bacillus* samples fall below the axis.

CI Positive Spectra

Under CI^+ conditions, the abundant M-18 EI fragment ions from unsaturated and cyclic free fatty acids were not present (Figures 4A–D). In the CI^+ spectra of *Y. pestis* (Figure 4B) and *B. melitensis* (Figure 4C), the $[M + H]^+$ ions of C18:1 and C19:1 FFAs were prominent at *m/z* 283 and *m/z* 297, respectively. Also, observed in the spectra of *Y. pestis* was the $[M + H]^+$ ion of C16:1 FFA at *m/z* 255 and the $[M + H]^+$ ion for cyC17:0 FFA at *m/z* 269 (Figure 4B). These two fatty acids were detected in all of the strains of *Y. pestis* examined (Table 2). The unique ion for *B. melitensis* that was observed at *m/z* 378 in EI became *m/z* 379 under CI^+.

Although the CI^+ spectra of the *Bacillus* species closely resembled their EI spectra in terms of observable fatty acids, there were some notable differences. In addition to fatty acid peaks, the CI^+ spectra of all three *Bacillus* species contained a peak at *m/z* 173. The appearance of a peak at *m/z* 173 was noteworthy as a previous study on the pyrolysis products of *Bacillus* cell wall extracts using methane CI^+ found an unknown peak at *m/z* 173 to be diagnostic for a particular strain of *B. anthracis* [10]. In our work, the peak at *m/z* 173 appeared in the spectra of both *B. cereus* and *B. anthracis*; however, the peak was much more predominant in *B. anthracis*. Chemical ionization spectra of a standard of poly(3-hydroxybutyrate) revealed that the source of *m/z* 173 was a dimer of this bacterial polyester. Poly(3-hydroxybutyrate) is thought to serve as an energy source for certain bacteria and spores and the thermal degradation products of poly(3-hydroxybutyrate) have been used as chemotaxonomic markers for bacteria [28–30]. Both *B. cereus* and *B. anthracis* contain poly(3-hydroxybutyrate) so it is thought that the observed differences in relative ion intensities are due to differences in growth conditions.

The $[M + H]^+$ ions from C16:0 (*m/z* 257) and C18:1 (*m/z* 283) FFAs were dominant in all three strains of *Francisella tularensis* examined, along with peaks from the C22:0, C22:1, C24:1, and C24:0 FFAs (Figure 4D) (Table 2). In addition, a peak at *m/z* 229 was observed in both the CI^+ and EI spectra of all *F. tularensis* strains examined with the relative intensity of *m/z* 229 being much greater in CI^+. The increased intensity of the peak at *m/z* 229 under CI^+ conditions is due to the contribution of the protonated C14:0 fatty acid which also has its $[M + H]^+$ ion at *m/z* 229.

Principal components analysis of the spectra obtained with CI^+ formed six distinct clusters in which all the spectra were grouped by sample class (Figure 2B). As in the EI mode, Gram-type differentiation was observed along the Component 1 score axis, with C15:0 (*m/z* 243) again being the dominant free fatty acid in the Gram-positive bacteria (Figure 3B). The loading plot for Component 1 contained the free fatty acids characteristic for each bacteria, such as *m/z* 269 from cyC17:0 in *Y. pestis* and *m/z* 243 from C15:0 in *Bacillus*. Contrary to EI results, the CI^+ PCA plot separated the three *Bacillus* samples along Component 1. As discussed earlier for the *Bacillus* and now observed statistically in the Component 1 loading, the distinguishing feature between the *Bacillus* samples were different relative intensities of C15:0 FFA at *m/z* 243 and the peak from poly(3-hydroxybutyate) at *m/z* 173. Component 1 contained 45% of the total variance and Component 2 contained 17%.

Negative Ion Spectra

When examining the whole bacteria by CI^- there was a visible increase in chemical noise which led to difficulty in obtaining good negative ion spectra. The spectra from whole bacteria were, in some cases, dramatically altered by slight changes in reagent gas pressure and were very susceptible to sample preparation conditions. In general, the negative ion spectra contained fewer peaks corresponding to free fatty acids than the EI or CI^+ spectra (Figure 5A–D). Those fatty acids that were observed were present as their $[M - H]^-$ ions. The lack of free fatty acid ions in negative ion mode is thought to stem from conditions in the ion source when whole bacteria are pyrolyzed. Changes in source pressure from the pyrolysis of whole bacteria could be the cause of the poor quality spectra in the negative ionization mode. As observed by others, the amount of water in the sample affected the quality of the CI^- spectra (Figure 6) [31].

Despite a reduced number of free fatty acid peaks, negative ion conditions did make it possible to readily observe the $M^{-\cdot}$ ion of dipicolinic acid (*m/z* 167) in sporulated samples (Figure 6). Dipicolinic acid is a chemical unique to sporulated bacteria and is often used to signal the presence of bacterial spores [7, 32].

As in CI^+, poly(3-hydroxybutyrate) is observed in the CI^- spectra of the *Bacillus* samples (Figures 5A and 6). The spectra of the *Bacillus* samples show $[M - H]^-$ ions from dimers and trimers of this bacterial polymer at *m/z* 171 and *m/z* 257, respectively. Also present are $M^{-\cdot}$ ions of trimers, tetramers, and pentamers at *m/z* 258, *m/z* 344, and *m/z* 430, respectively.

Principal components analysis of the negative ion data resulted in the formation of three clusters with *Y. pestis* and *B. melitensis* grouped together and *B. cereus* vegetative and *B. anthracis* vegetative also being grouped closely (Figure 2C). The spreading out of the *Bacillus* samples due to poly(3-hydroxybutyrate) roughly follows what was observed in the CI^+ PCA plot. This result is not surprising since this polyester is detected in both CI^+ and CI^- spectra. The spectra of sporulated *B. anthracis* were easily separated from the other bacteria due to the $M^{-\cdot}$ ion of dipicolinic acid at *m/z* 167 and an unknown ion at *m/z* 181. Although not as clear as in the other two ionization modes, Gram-type differentiation was still defined by the Component 1 score axis. The loading plot of Component 1 revealed that bacteria Gram-type differentiation resulted from ions corresponding to poly(3-hydroxybutyrate) and di-

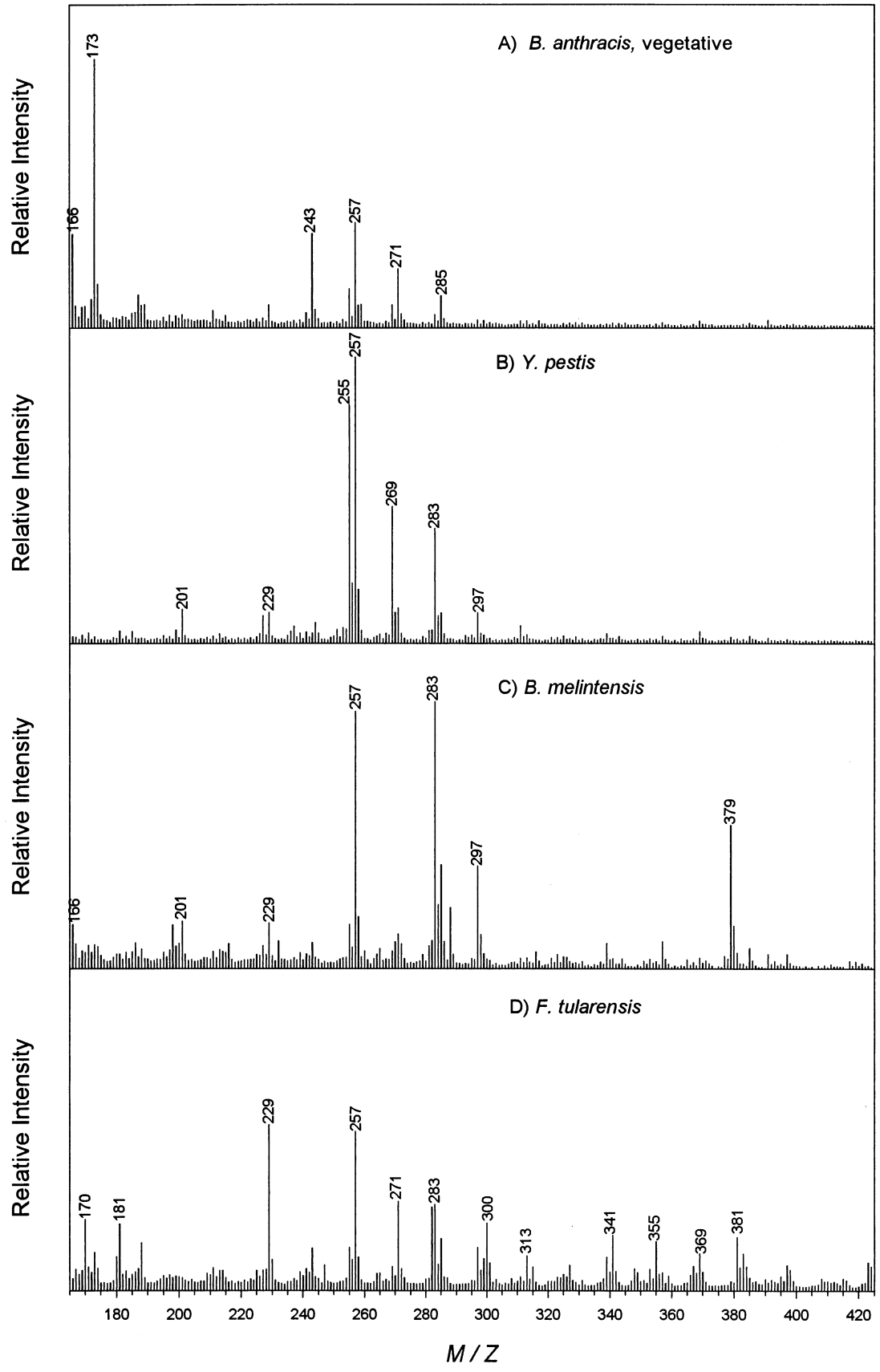

Figure 4. CI^+ spectra of selected whole bacteria.

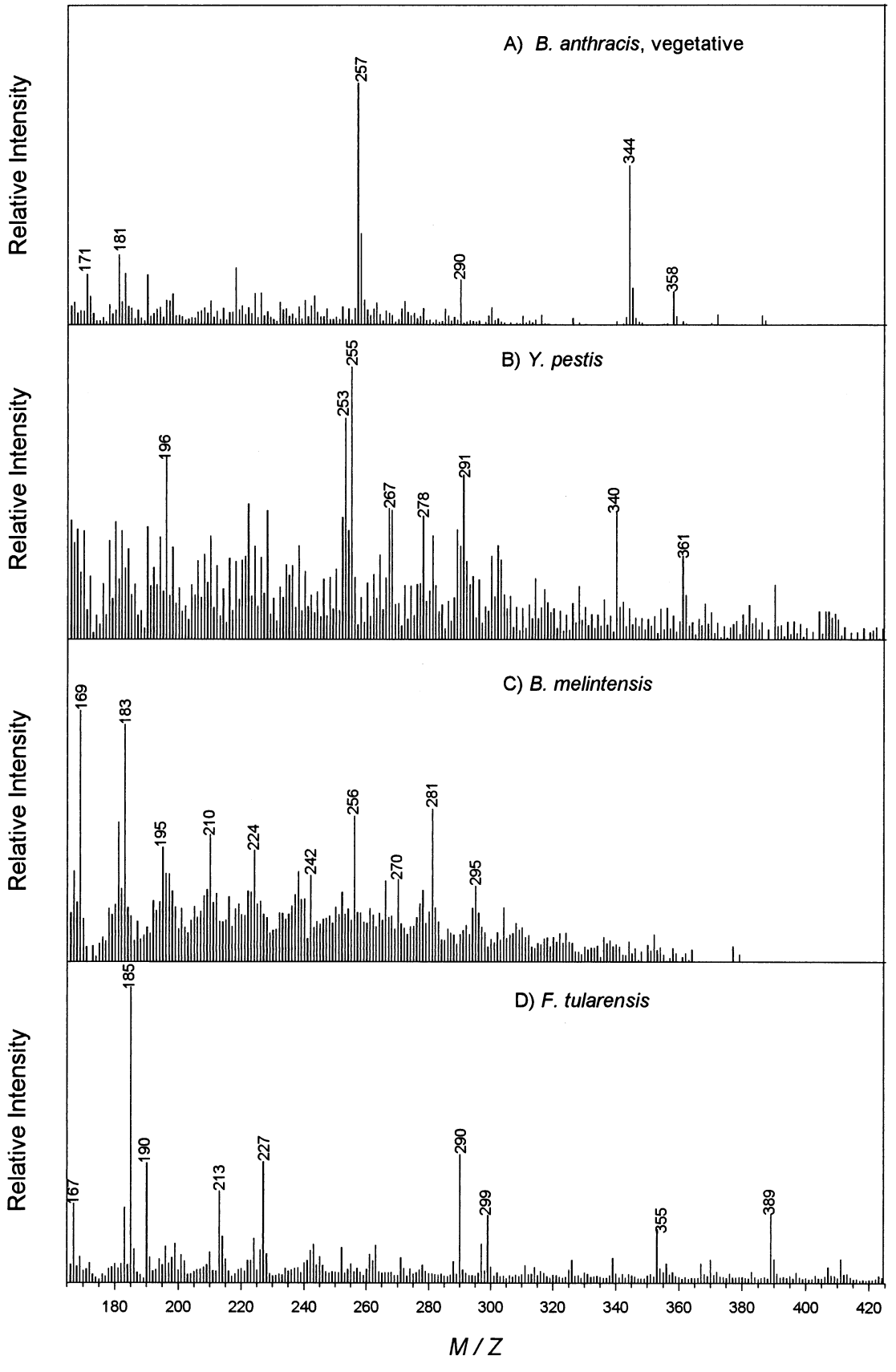

Figure 5. CI^- spectra of selected whole bacteria.

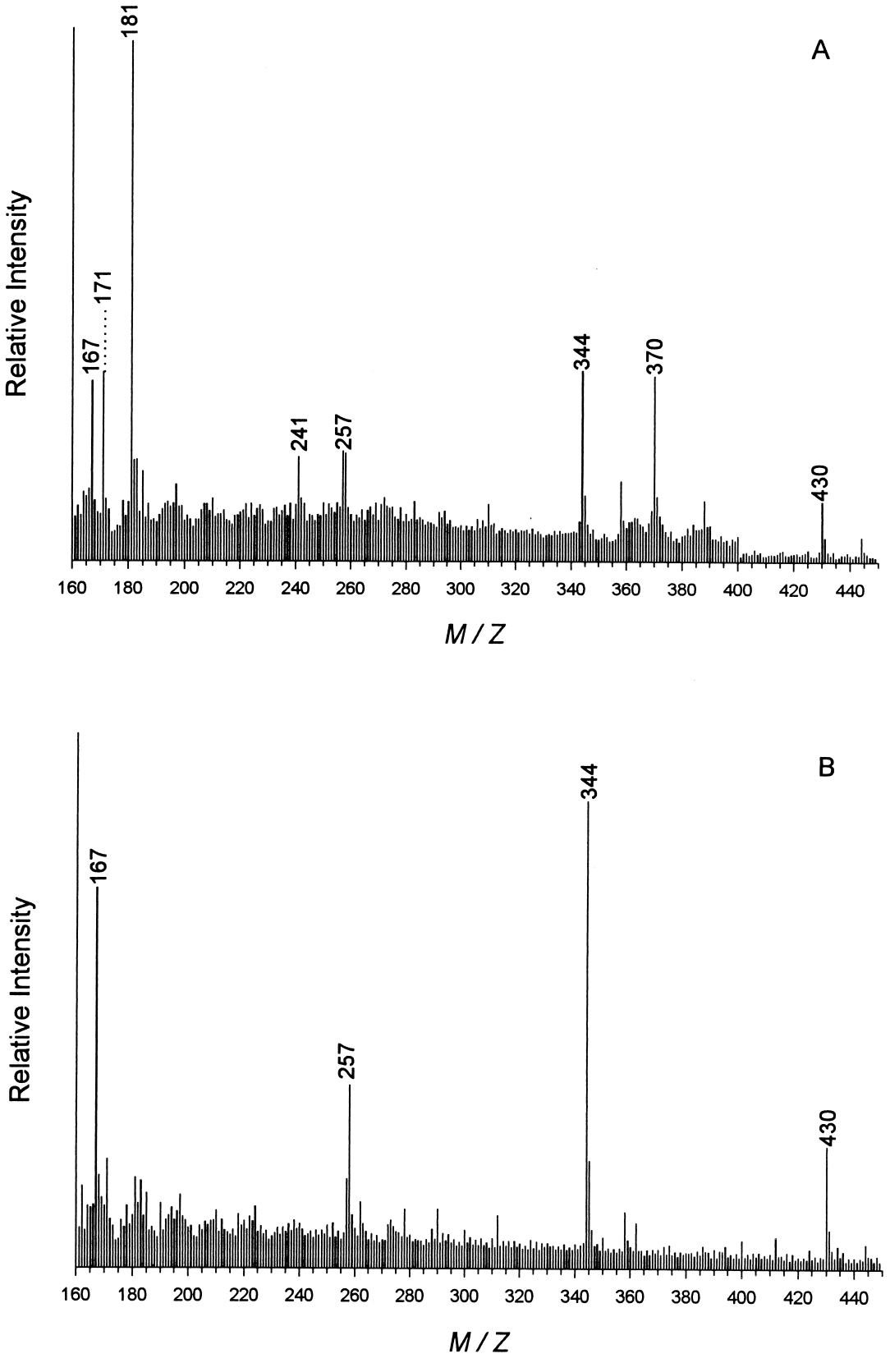

Figure 6. CI^- spectra of sporulated *B. anthracis* after air drying for (**A**) 2 h and (**B**) overnight.

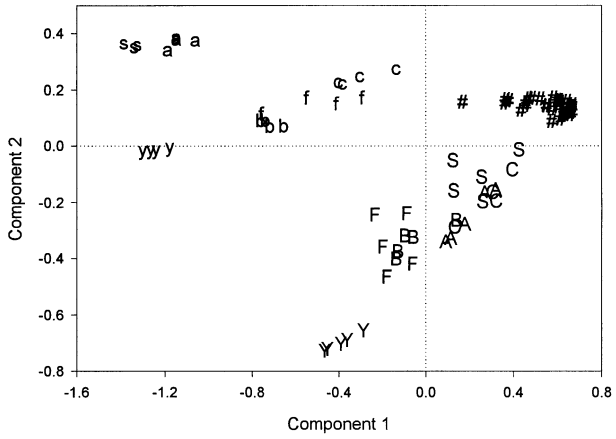

Figure 7. PCA score plot of whole bacteria spectra obtained under all ionization modes. Uppercase letters = EI spectra; lowercase letters = CI^+ spectra; number symbols = CI^- spectra.

picolinic acid (Figure 3C). Component 1 contained 34% of the total variance and Component 3 contained 14%.

PCA Comparison of Ionization Modes

The spectra from all three ionization modes were combined into a single PCA score plot to provide better insight into which ionization mode was able to produce the optimal clustering and differentiation among the bacterial species. A direct comparison of this type was possible as all the spectra were collected over the same mass range and all the peaks in that mass range were used for the PCA. Figure 7 is the PCA score plot of the spectra produced from EI, CI^+, and CI^- ionization modes. The spectra obtained in negative ion mode, designated as number symbols (#), are tightly grouped together with no discernment between the six sample classes. Such a result is not surprising when the component axes of the CI^- PCA score plot are examined. The variance scales of the component axes for the CI^- PCA score plot are smaller than their EI and CI^+ counterparts, hence the tighter grouping of CI^- spectra when compared to EI and CI^+. The CI^+ spectra, plotted as lowercase letters, produced the greatest distance between clusters. The separation of the three *Bacillus* samples that was observed in the individual CI^+ PCA score plot was retained in the combined PCA score plot. Spectra obtained under 70-eV EI, plotted as uppercase letters, had sample classes separated by distances somewhere between those observed for the CI^+ and CI^- spectra.

Using lipid information only in the analysis, CI^+ spectra produced PCA results comparable to EI, despite containing greater molecular ion intensity and less fragmentation. When PCA was carried out using *only* the molecular ions for the fatty acids listed in Table 3, EI and CI^+ data produced the same clustering of samples with similar intra- and inter-class distances. Neither ionization mode could separate the *Bacillus* samples from one another when only the fatty acid ions in Table 3 were used in the PCA. The major factor that caused the CI^+ spectra to be better differentiated under PCA than the EI spectra was that taxonomically significant peaks (other than fatty acids) were observed under CI^+ conditions that were not present in EI.

Mixtures of Bacteria

The benefit of having unique ions in the mass spectra of some of the bacteria is demonstrated when a mixture of four bacteria is analyzed under 70-eV EI and CI^+ conditions (Figure 8A, B). A bacterial mixture was made up using 2-μL bacterial suspensions (10 mg/mL) from *B. anthracis* vegetative, *B. melitensis*, *F. tularensis*, and *Y. pestis*. Only peaks that are unique to the spectrum of a particular bacteria are labeled in Figure 8.

The EI spectrum of the mixture contained peaks easily identified with *B. melitensis* at *m/z* 339 and *m/z* 378. The peaks for the C24:1 and C24:0 FFAs at *m/z* 348 and *m/z* 368 and the peak at *m/z* 229 were associated with *F. tularensis*. Also observed, but less prominent, were the EI fragment ions at *m/z* 236 and *m/z* 250 associated with *Y. pestis*. The remainder of the peaks in the EI spectra were difficult to correlate with a particular bacteria and could have originated from any of the four bacteria examined. For example, the peak at *m/z* 264 was present in the EI spectra of both *Brucella* and *Y. pestis* and no peaks in the EI spectra were unique to the *Bacillus* samples.

The CI^+ spectrum of the bacterial mixture was easier to interpret than the EI spectrum since it contained unique $[M + H]^+$ fatty acid ions associated with each genera of bacteria plus the signal-to-background ratio of all ions was greater than in the EI spectra (Figure 8B). Observed in the CI^+ spectrum as their $[M + H]^+$ ions was the peak at *m/z* 379 associated with *B. melitensis* and two peaks characteristic of *F. tularensis* at *m/z* 341 and *m/z* 369 from the C22:0 and C24:1 FFAs. Detected only under CI^+ conditions were the peaks at *m/z* 269 and *m/z* 255 associated with *Y. pestis* and the peak at *m/z* 173 indicating the presence of a *Bacillus* species. As mentioned earlier, the discriminating feature between the *B. anthracis* and *B. cereus* CI^+ spectra were differences in peak ratios; thus the appearance of the peak at *m/z* 173 can only signal the presence of a *Bacillus* species and not provide information about the individual species. A negative ion scan for DPA would, however, determine if the *Bacillus* species was sporulated.

Conclusion

Both EI and CI^+ conditions produced fatty acid profiles that contained comparable lipid taxonomic information. However, CI^+ produced peaks not observed in EI that, in some cases, had a significant impact on the taxonomic profiling of the spectra. For example, the $[M + H]^+$ ions from poly(3-hydroxybutyrate) at *m/z* 173, *m/z* 259, and *m/z* 345 served as a marker for the *Bacillus*

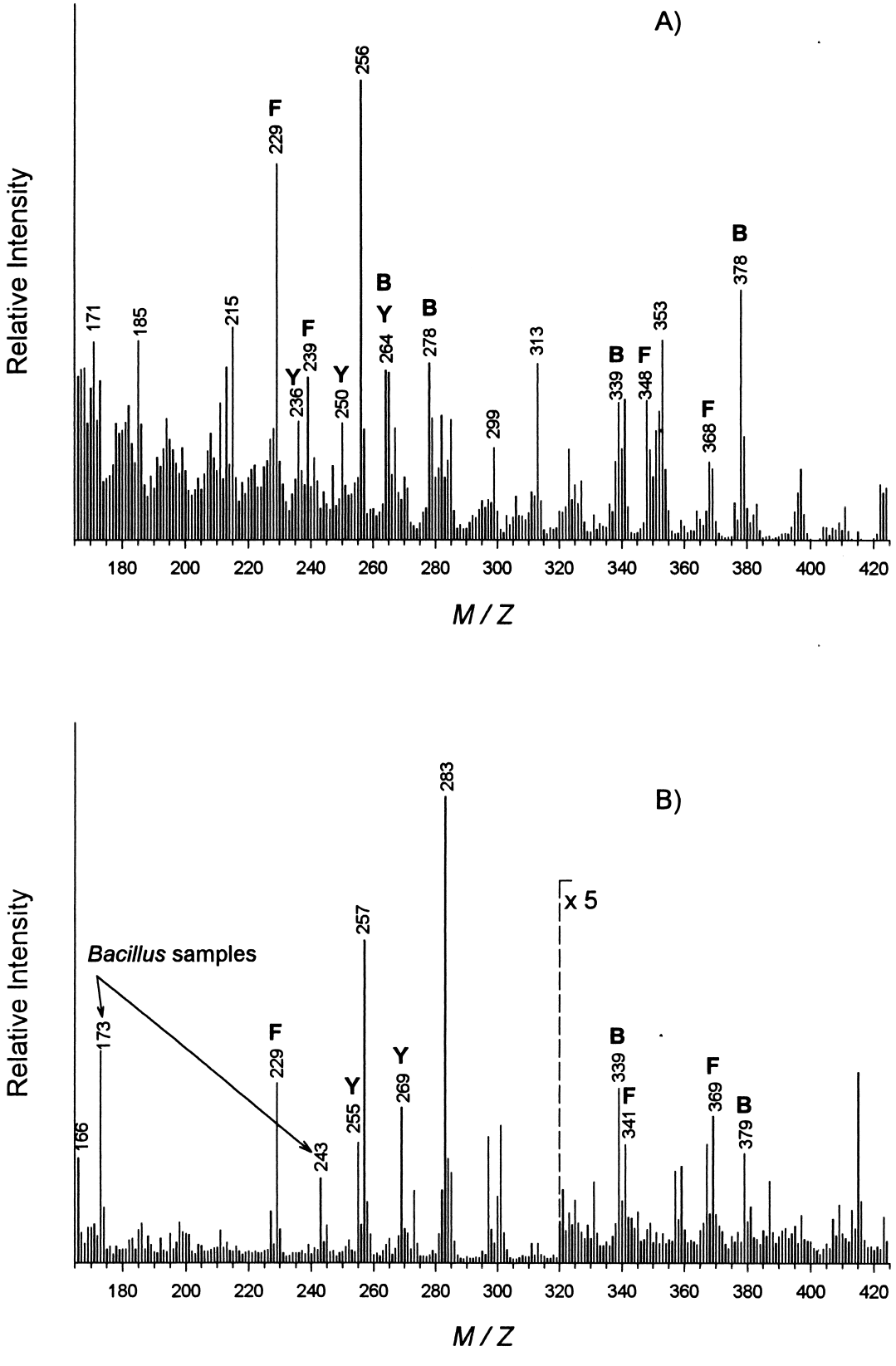

Figure 8. Mixture of *B. anthracis* (vegetative), *B. melitensis*, *Y. pestis*, and *F. tularensis*. (**A**) 70-eV spectra of mixture, (**B**) Isobutane CI^+ spectra of mixture. Peaks associated with a specific bacteria are labeled **F** = *F. tularensis*, **Y** = *Y. pestis*, **B** = *B. melitensis*.

species in CI^+ mode. The peak at *m/z* 173 was particularly prevalent in the *B. anthracis* spectra, a result previously found by Adkins et al. [10]. It is the observance of unique ions under CI^+ conditions that allowed for CI^+ to achieve a greater differentiation with PCA.

Preliminary investigations with other strains of *Y. pestis*, *F. tularensis*, and *B. melitensis* indicated that the diagnostic peaks observed for these bacteria in CI^+ are independent of strain and growth conditions. This result complements a recent report on the direct EI MS lipid profiling of these bacteria [8]. Although the taxonomic significance of these ions only apply to this suite of bacteria, similar findings on the benefit of CI^+ to bacterial MS lipid profiling have recently been presented by researchers using an ion trap MS to examine bound and free bacterial fatty acids [33].

Compared to EI and CI^+, CI^- conditions produced spectra that had fewer fatty acid peaks. Despite containing less information for the lipids, some taxonomically significant compounds, such as dipicolinic acid and poly(3-hydroxybutyrate), were readily observed with this ionization mode. It is possible that the selectivity and sensitivity of negative ion chemical ionization could be used advantageously to detect specific bacteria containing these taxonomic compounds.

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